REMARKS

Claims 1-10 had been pending in the present application. As in the parent case, Claims 1-6 are withdrawn from consideration. Merely to facilitate examination, Claims 7-10 have been cancelled and new Claims 11-14 introduced in their place. These claims are now in the form presented in the Response and Amendment of Jan. 27, 2004 in the parent application, except that claims 11, 12, and 14 (formerly claims 7, 8, and 10) now recite ". . . a sulfur atom-free enzyme protein that retains activity." This amendment is supported in the specification, for example, at page 6, lines 3-6. Thus, Applicant respectfully requests entry of the amendment.

I. Rejection of Claims 11-14 (formerly 7-10) Under 35 U.S.C. § 112, first paragraph

The Office rejected claims 7-10 under 35 U.S.C. § 112, first paragraph, as allegedly lacking enablement. Final Action at page 2. Specifically, the Office stated that:

> the specification, while being enabling for methods of construction [of] the sulfur-free DHFR genes of Table 7 of the specification and a sulfur-free xylanase gene encoding SEQ ID NO:9, does not reasonably provide enablement for methods of constructing any sulfur-free enzyme having activity greater than or equal to the activity of the original protein.

Id.

Solely to expedite prosecution and without acquiescing to the Office's rejection, Applicant proposes to amend claims 11, 12, and 14 to recite ". . . a sulfur atom-free enzyme protein that retains activity." "Retains activity" is defined in the specification as "not less than 10% activity." Specification, e.g., at page 6, lines 3-6. Thus, a sulfur

atom-free enzyme protein produced by any of the claimed methods need not have activity "greater than or equivalent to that of the original enzyme protein."

Furthermore, the basis for the Examiner's rejection is that "the skilled artisan would not know which enzymes the disclosed methods would be applicable to and the specification, fails to provide any guidance for selecting those enzymes that could be reasonably expected to succeed." Final Action at pages 3-4. In a telephonic interview with the undersigned on July 20, 2004, the Examiner further asserted that enzymes containing disulfide bonds or active site cysteine residues would not be expected to possess activity if subjected to the claimed methods. *See* Applicant Statement of Interview Summary at pages 1-2, filed herewith. Thus, the claims are allegedly inoperative with respect to such enzymes.

The scope of the claims may include inoperative embodiments so long as one skilled in the art can determine which embodiments would be inoperative without undue experimentation. *See* M.P.E.P. § 2164.08(b) at 2100-199 (8th ed. rev. 2, May 2004). The Examiner's own arguments demonstrate that the knowledge in the art at or before the time of filing provides sufficient guidance as to inoperative embodiments. For example, one skilled in the art would expect that an enzyme having an active site cysteine residue that acts as a nucleophile might not retain activity if that residue undergoes substitution. *See, e.g.*, attached article by Takagi et al. (1998) Proc. Natl. Acad. Sci. USA 95:9808-9812, at page 9808 (abstract; col. 1, ¶ 2; and col. 2, ¶ 1) (showing that substitution of a nucleophilic active site cysteine residue with a serine residue results in an inactive enzyme). Similarly, one skilled in the art would expect that an enzyme having critical disulfide bonds might not retain activity if the cysteine

residues participating in those disulfide bonds undergo substitution. *See, e.g.*, attached article by Plano et al. (1991) J. Bacteriol. 173:3389-3396, at page 3392, paragraph bridging cols. 1 and 2 (showing that maintenance of a critical disulfide bond is necessary for enzyme activity). Thus, one skilled in the art could determine inoperative embodiments without undue experimentation.

Because claims 11-14 are fully enabled, Applicant requests withdrawal of this rejection under 35 U.S.C. § 112, first paragraph.

II. Rejection of Claims 11-14 (formerly 7-10) Under 35 U.S.C. § 103(a)

In the Final Action, the Examiner stated that if she withdrew the rejection of Claims 11-14 (formerly Claims 7-10) under 35 U.S.C. § 112, first paragraph, she would reinstate the rejection of those claims under 35 U.S.C. § 103(a) as being unpatentable over the combined disclosures of Li et al. (1995) *Biotech. and Bioeng.* 48:490-500, and Recktenwald et al. (1993) *J. Biotech.* 28:1-23, in view of Lathrop et al. (1992) *Prot. Exp. and Pur.* 3:512-17, and Barnett et al., WO 96/30481. *See* Final Action at page 5; *see also* Applicant Statement of Interview Summary at page 2. During the telephonic interview of July 20, 2004, the Examiner further asserted that the cited art discusses replacing sulfur-containing amino acids that are particularly labile in alpha-amylase in order to confer stability to the enzyme. *Id.* The Examiner concluded that substitution of the remaining sulfur-containing amino acids, which are not important for enzyme stability, would be obvious in view of such methods. *Id.*

Applicant respectfully traverses that rejection. Applicant reiterates the arguments made previously which are incorporated by reference. *See* Applicant's Response and Amendment of Jan. 27, 2004 at p. 16 - 18. Applicant contends, as before, that the

combined disclosures of Li et al. and Recktenwald et al. do not teach or suggest replacing all methionine and cysteine residues in a single protein. *See id.* at p. 16. Rather, the combined disclosures teach the selective replacement of particular methionine or cysteine residues based on rational criteria and, therefore, teach away from replacing all methionine and cysteine residues in a single protein.

First, Li et al. teach that local protein structure determines susceptibility of amino acids to oxidation and that it is desirable to focus on only the oxidizable amino acids. Li et al., *Biotech. and Bioeng.* 48 at 493-94. For example, Li et al. state that "the amino acid residues which are located in buried positions within a protein are less easily oxidized by exogenously added oxidants . . . . For example, human growth hormone contains three methionine residues in the molecule: Met-14, Met-125, and Met-170. The reactivities of the three methionine residues toward H_2O_2 are shown to be directly correlated to their solvent-accessible surface areas." *Id.* at 494. Li et al. conclude that the surface residues Met-14 and Met-125 are readily oxidizable whereas Met-170, which is in a buried position, is unoxidizable. *Id.* Li et al. also describe the oxidation of only two of 28 tryptophan residues in IgG. *Id.* Thus, Li et al. teach that only a few of the sulfur-containing amino acids in a protein are actually oxidizable, that susceptibility to oxidation is determined by rational criteria such as location on a solvent-accessible surface, and that the selected ones are replaced. This reference suggests mutating only the oxidation-susceptible amino acids, thus teaching away from mutating *all* sulfur-containing amino acids in a single protein.

Moreover, Li et al. disclose the desirability of replacing as few amino acids as possible in a protein. "Whereas site-directed mutagenesis may be used to increase

chemical stability, its effects on conformation, biological activity and immunogenicity can be significant, and sometimes quite undesirable." Li et al., *Biotech. and Bioeng.* 48 at 497. Thus, this reference teaches that site directed mutagenesis may adversely affect biological activity, among other effects. Accordingly, the lesson one takes from Li et al. is to selectively replace only those amino acids which are known to be oxidizable, such as those that are on the protein surface. There is no suggestion in this reference that mutation of all sulfur-containing amino acid residues in a single protein is desirable or that such a multiple mutant will retain biological activity. Indeed, it is the present inventor who has shown that the claimed methods result in a sulfur-free enzyme protein that retains activity.

In addition, as noted previously, while Recktenwald et al. teach the substitution of methionine, cysteine or tryptophan as targets for protein engineering, the substitution of all methionine and cysteine residues in a single protein is not discussed. *See* Applicant's Response and Amendment of Jan. 27, 2004 at p. 17. Moreover, Recktenwald et al. describe how substitution by site-directed mutagenesis is to be informed by structural data available for the protein. According to Recktenwald et al., "[b]ased on the 3-D structure, and on assumptions on how to manifest the desired change in the protein - possibly tested by computer graphics - the cloned gene is altered by site-directed mutagenesis." Recktenwald et al., *J. Biotech.* 28 at 7. Recktenwald et al. teach site-directed mutagenesis of selected amino acids, stating that "computer modeling is essential . . . [to] . . . identif[y] . . . those positions in the protein sequence that should be changed, and predict[ ] . . . the result this change in primary structure has on 3-D structure, activity and stability of the protein." Recktenwald et al.,

J. Biotech. 28 at 6. Thus, according to this paper, selective amino acid substitution can avoid affecting protein structure, activity or stability. As also noted by Li et al., such "effects on conformation, biological activity and immunogenicity can be significant, and sometimes quite undesirable." Li et al., *Biotech. and Bioeng.* 48 at 497. Thus, while the combination of Li et al. and Recktenwald et al. may teach the substitution of single methionine, cysteine, or tryptophan residues based on rational protein design principles, the combined references teach away from the substitution of all sulfur-containing amino acid residues in a single protein.

As the combination of Li et al. and Recktenwald et al. fails to teach or suggest substituting all methionine and cysteine residues, there is no motivation to use the methods set forth in Barnett et al. and Lathrop et al. Applicant's Response and Amendment of Jan. 27, 2004 at p. 17. The Office states that Barnett et al. teach the site-specific mutation of oxidizable amino acids to increase oxidative stability including the mutation of multiple amino acids within a single protein. Office Action, Aug. 27, 2003 at p. 9. However, just as did Li et al. and Recktenwald et al., Barnett et al. teach selective replacement of oxidizable amino acids, followed by the combination of single mutants previously shown to have desired properties to construct multiple mutants. However, Barnett et al. do not teach the replacement of *all* sulfur-containing amino acids in a single protein by a combination and/or stepwise mutation method as explained further below.

Examples 1, 2, 4, and 10 in Barnett et al. illustrate the selective replacement approach. First, Barnett et al. constructed a set of single replacement mutants where each one of the seven methionine residues in alpha-amylase were

replaced (example 1 at p. 16-17) and tested each variant for susceptibility to oxidation (example 2 at p. 19-20). "Variants at six of the methionine positions in *B. licheniformis* alpha-amylase were still subject to oxidation by peroxide while the substitution at position +197 (M197L) showed resistance to peroxide oxidation." Barnett et al., example 2 at p. 20. In addition, a second variant with a modified methionine residue at position +15 exhibited increased performance in a starch liquefaction assay (example 4 at p. 25-26). Throughout the entire Barnett et al. disclosure, the only methionine mutants chosen for further study and/or for the construction of multiple mutants are Met197 and Met15, each of which had only one methionine mutated, at either position +15 or +197. The remaining five single methionine mutants exhibiting no desirable properties were not studied further and were not used for the construction of multiple mutants. Thus, Barnett et al. teach the selective replacement of methionine residues, not the replacement of *all* methionine residues in a single molecule.

In addition, the Office states that "Barnett et al. teach specific methods of constructing multiply substituted site-specific mutants which include a) constructing all possible single site mutants at several distinct methionine residues, selecting those single mutants with the highest activity and then screening combinations of these mutations for those multiple mutants with the highest activities . . . and b) creating double mutants by further mutating a precursor single mutant enzyme." Office Action, Aug. 27, 2003 at p. 9-10. The Office asserts that method a) includes the features of steps (2) - (4) of Claim 11 (formerly Claim 7) and method b) includes all the features of steps (2) - (4) of Claims 12 and 13 (formerly Claims 8 and 9). Office Action, Aug. 27, 2003 at p. 10. Although some multiply substituted site specific mutants are created,

they are created by the selective replacement of one or more oxidizable residues, and then by the combination of previously screened and selected single mutants. Barnett et al. do not teach or suggest a combined and/or stepwise mutation method leading to replacement of all sulfur-containing residues in a single protein as claimed in steps (2)-(4) of Claim 11 (formerly Claim 7) and steps (2)-(4) of Claims 12 and 13 (formerly Claims 8 and 9).

To further elaborate, Example 10 teaches selective replacement of a second amino acid in a protein for construction of a double mutant. First, by studying the oxidative susceptibility of the alpha-amylase single methionine mutant at residue 197 to chloramine-T, Barnett et al. suspected that tryptophan residue 138 was oxidized by chloramine-T. "Tryptic mapping and subsequent amino acid sequencing indicated that the tryptophan at position 138 was oxidized by chloramine-T. To prove this, site-directed mutants were made at tryptophan 138." Barnett et al., example 10 at p. 33.

Then, Barnett et al. constructed double and triple mutants where only those amino acids previously determined to improve oxidative stability were replaced, specifically Met15, Met197 and Trp138. Double and triple mutants of only these three particular amino acids were tested in example 11, and the best mutant, a triple mutant containing a replacement at Met15, Met197 and Trp138, was tested in examples 14, 16 and 17. This triple mutant of alpha-amylase contained replacements at just two of seven methionine residues and only one of 17 tryptophan residues (*see* fig. 2).

Thus, the methods of Barnett et al. teach the construction of multiple mutants by selective replacement. Although a set of single methionine replacement mutants were created and tested, not all of the single methionine mutants were

combined to make a multiple mutant lacking *all* sulfur-containing amino acids. Thus, Barnett et al. do not teach or suggest a combined and/or stepwise mutation method leading to replacement of all sulfur-containing residues in a single protein as claimed in steps (2)-(4) of Claim 11 (formerly Claim 7) and steps (2)-(4) of Claims 12 and 13 (formerly Claims 8 and 9).

Finally, the Office states that Lathrop et al. provides a method for removing the initiator methionine, and that this reference combined with Barnett et al. provides a method for removing all methionine residues from a protein. Office Action, Aug. 27, 2003 at p. 11. Applicant asserts, as before, that while Lathrop et al. discuss substituting the amino acid following the initiator methionine to allow removal of the initiator methionine by aminopeptidase, they do not discuss substituting all methionine residues. Applicant's Response and Amendment of Jan. 27, 2004 at p. 18. As asserted previously, and as elaborated upon in the discussion above, the art provides no motivation to combine these references. Therefore, Li et al. and Recktenwald et al. fail to render the claims obvious in view of Barnett et al. and Lathrop et al.

In view of the foregoing remarks, Applicant requests withdrawal of the rejection of Claims 11-14 (formerly Claims 7-10) under 35 U.S.C. § 103(a), if the Examiner reinstates that rejection.

CONCLUSION

Applicant respectfully asserts that the application is in condition for allowance. If the Examiner does not consider the application to be in condition for allowance, Applicant requests that the Examiner call the undersigned at (650) 849-6778 to arrange an interview prior to taking action.

Please grant any extensions of time required to enter this Amendment and Response and charge any additional required fees to Deposit Account No. 06-0916.

Respectfully submitted,

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Dated: February 3, 2005

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